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Incidence of Aflatoxigenic Fungi and Aflatoxins in Maize Cultivated Under Rain-Fed and Irrigation Farming Systems in Kenya

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Authors' contributions

This work was carried out in collaboration between all authors. Author SC designed the study, wrote the protocol, performed statistical analysis and wrote the first draft of the manuscript. Authors WW, CB and DO managed literature survey and analyses of study. Authors SC and CB identified the fungal species. Authors SC and JG managed aflatoxin ELISA spectrophotometry assays. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Aims: This study was undertaken with the aim of establishing whether irrigation reduces aflatoxigenic fungal and aflatoxin contamination of maize samples purposefully selected from two regions practicing rain-fed and irrigation farming systems Kenya.

Place and Duration of Study: Rain-fed maize samples were obtained from Kitui and Kibwezi districts while irrigation samples were from Perkerra Irrigation scheme in Baringo County. Moisture content and fungal contamination analysis was undertaken at the Centre for Biotechnology and

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Bioinformatics, University of Nairobi during 2008-2010 periods. Analysis for aflatoxin contamination was done at BORA Biotech Ltd laboratories, Nairobi.

Methods: A total of 155 maize samples were obtained from two regions practicing Rain Fed Farming System and Irrigation Farming System. A purposeful sampling technique was used during the two year study. Aflatoxigenic fungal contamination involved culture on modified Potato dextrose agar supplemented with Yeast Extract Sucrose Agar containing 0.3% β -cyclodextrin. Aflatoxin quantification was done by Enzyme-Linked Immunosorbent Assay (ELISA). The findings from these two variables were compared according to the farming system. Correlation analysis between the grains moisture content, fungal and aflatoxin contamination was undertaken.

Results: Significant difference existed in the mean grains' moisture content (M.C.) of maize samples from the two farming systems (P < 0.001). The mean moisture content was 13.2% and 12.5% for Rain Fed and Irrigation Farming System samples, respectively. A significant relationship was established in the variation and frequency of aflatoxigenic fungal species. Aspergillus flavus occurred predominantly in Rain Fed samples ($X^2=16.764$, p=0.05). The proportion of samples with both fungal and aflatoxin contamination was comparatively higher among Rain Fed than Irrigation samples with aflatoxin contamination in 73.7%, and 59%, of the samples, respectively. However, difference in the mean fungal and aflatoxin contamination according to farming system was insignificant. A positively weak correlation existed between the total fungal load and aflatoxin levels in maize samples from both farming systems ($R^2=0.041$ and $R^2=0.004$, respectively).

Conclusion: Use of irrigation, certified maize seed varieties, adequate maize grain drying and sound farming practices contribute to lower fungal and aflatoxin contamination.

Findings from this study are of great significance in creating awareness on the need to modify the pre-harvest and post-harvest farming practices in the various irrigations schemes that the Kenyan government continues investing billions of money in revamping. These practices will ensure that the maize harvests do not go into waste due to fungal and aflatoxin contamination thereby contributing to creation of national food safety and security.

Keywords: Aflatoxigenic fungi; aflatoxins, maize; varieties; rain fed; irrigation; farming system.

1. INTRODUCTION

Maize is a staple food among the urban and rural households in Kenya with an average consumption rate of 0.5 Kg/person/day. It is also used as a raw material for various products including animal feeds, corn oil, starch powder and traditional liquors. The annual production is approximately 36 million bags each of 90 Kg. Trans-Nzoia, Uasin-Gishu and Nandi counties contribute about 90% of the national output [1,2]. This production is mainly under rain-fed farming system. However, with 80% of the country being arid and semi-arid with annual rainfall averages 400 mm, the national maize production hardly attains the national per capita consumption. This deficit compromises the national food safety whereby in bid to avert hunger, aflatoxin contaminated maize meal forms regular affordable diet.

Aflatoxigenic fungi and aflatoxins were the primary focus of this study due to its prevalence with recurrent fatal aflatoxicoses outbreaks. It is therefore a significant challenge to the National food security and public health. Kenya, to date, is the only nation worldwide with a population that has repeatedly experienced deadly epidemics of acute aflatoxicoses over the past three decades including; 1982, 2001, 2004 and 2005 [3-7]. These outbreaks resulted from consumption of highly contaminated homegrown maize. The most recent outbreak occurred from 2004 through 2006, when several hundred Kenvans died from acute aflatoxin poisoning in several districts of the Eastern Province including Machakos, Makueni, Kitui and Mbeere [8,9]. During these periods, many thousands of individuals were exposed to unsafe aflatoxin levels, with 317 cases reported and 125 deaths [10]. Other than these fatal aflatoxicosis episodes, by virtue of dietary preferences for maize among Kenyans and the carcinogenic nature of aflatoxins, consumption of suboptimally aflatoxin contaminated maize meals poses greater public health hazard relative to the acute cases that is usually given high publicity sporadically [11]. For instance, despite no reports of acute aflatoxicosis in the country in the last three years, maize grain harvest and maize in urban markets including Nairobi have been found to have dangerously high aflatoxin levels [12-15].

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Food security and food safety is now a major concern in Kenya particularly in view of attainment of developmental goals of vision 2030. Due to the strategic importance of maize as a staple food, the government rolled out a plan to produce maize and rice under irrigation to enhance the national grain strategic reserves under the National Economic Stimulus Project [16]. Billions of funds have been used to revamp various irrigation schemes particularly Bura, Hola and Perkerra irrigation schemes that cultivate mainly maize. Maize harvests from some of these irrigation schemes have however, been reported to have aflatoxin levels beyond the statutory levels recommended of 10 ppb for total aflatoxins [17-19]. In the previous studies undertaken in Kenya on aflatoxigenic fungi and aflatoxins in maize, little attention has been given to the role of irrigation farming system in reducing aflatoxin contamination. The only research work established that high temperature and periodic drought prevalent in the semi-arid regions of Kenyan accounted for the higher levels of A. flavus and AflatoxinB1 contamination compared to regions with higher rainfall and humidity [7,20]. However, no studies to date have been undertaken to compare incidence of aflatoxigenic fungi and aflatoxins in maize grain cultivated under rain-fed and irrigated farming systems in Kenya especially considering that drought, high temperatures and poor soil fertility have been found to greatly contribute to preharvest mycotoxin contamination of food crops [21].

This study therefore aimed to undertake a comparative a study on the contamination of maize cultivated under these farming systems in two regions with similar agro-ecological conditions in Kenya.

2. MATERIALS AND METHODS

2.1 Sampling Sites and Sample Collection

The study areas were Perkerra Irrigation scheme in Baringo county and two counties historically associated with aflatoxin outbreaks, in Kenya, namely Kitui county and Kibwezi sub-county. The two study regions were chosen on the basis of type of farming system, specifically whether rainfed rainfall or irrigation and also their past records of aflatoxicosis outbreaks. The two sampling sites have closely similar agroecological conditions, ambient temperature and the annual rainfall amount where both sites are ecologically classified as semi-arid regions. However, while maize cultivation is undertaken under irrigation system in Perkerra Scheme, farming is under rain-fed rainfall in Kitui and Kibwezi counties. Further, while Marigat division of Baringo County, the location of Perkerra irrigation scheme, has never had reported aflatoxicosis outbreaks, Kitui and Kibwezi districts have had a long aflatoxicosis history spanning the last thirty years. Sampling of maize grain was undertaken during the period 2008-2010 whereby a total of 61 and 94 samples were obtained from Perkerra irrigation scheme and Kitui/Kibwezi counties. respectively. А representative minimum working sample of 2-kg was obtained at each sampling stage. The samples were packed in paper bags and stored at 4°C until further analysis which was carried out within 72 hours. Fungal isolation was done at Centre for Biotechnology and Bioinformatics (CEBIB), University of Nairobi while Aflatoxin analysis was done at BORA Biotech Ltd laboratories, Nairobi.

2.2 Determination of Grains' Moisture Content

The 2 Kg of each representative sample maize grain was ground under aseptic condition of 70% ethanol into fine flour using Wiley Mill No.1 at National Public Health Laboratories, Nairobi. The moisture content of each sample of maize grain was determined using the hot-air oven method [22].

2.3 Isolation and Identification of Aflatoxigenic Fungal Species

The aflatoxigenic fungal spp. targeted for isolation from the maize samples in this study were Aspergillus flavus and A. parasiticus. Modified Potato Dextrose Agar (PDA) enriched with Yeast Extract Sucrose (YES) agar containing 0.3% β-cyclodextrin was used for isolation. Dilution plating technique described for isolation of fungi from powdered foods was used [23]. Triplicates of 10 g of each powdered subsample was diluted in 100 ml of sterile double distilled water and vortexed for 1 min. Appropriate dilutions of 1 ml of the mixture were inoculated on a set of triplicate agar plates using sterile glass spreader. The plates were finally incubated without illumination at 30°C for 3 days. After incubation, isolates were identified as A. flavus and A. parasiticus according to colony color and conidia surface texture features [23-25]. Fungal load in each maize samples was expressed as Colony Forming Units per Unit weight in grams (CFU/g).

2.4 Determination of Aflatoxin Contamination in Maize Samples

Aflatoxin levels in the maize samples were determined by direct competitive enzyme linked ELISA commercial Kit, BORA® from BORA Biotech, Kenya. The method has a lower detection limit of 2 µg/kg [26,27]. Briefly, the method involved aflatoxin extraction from portion of 5 g finely ground sub-sample using methanol/water solution. The extract was then defatted with hexane and centrifuged followed by supernatant recovery and dilution with buffered saline (PBS). The resulting solution was further diluted with methanol-PBS mixture before aflatoxin quantification on ELISA microtiter plates. The wells of ELISA plate were prior coated with anti-aflatoxin antibody, incubated overnight in a moist chamber then emptied. Any free protein binding sites were blocked using bovine serum albumin in PBS followed by plate washing with Tween 20 solution and semi-dried. Volumes of sample extract and equal volumes of AFB1 standards were added into separate wells. Solution of AFB1-enzyme conjugate was simultaneously added to all wells before 2 hour incubation in darkness followed by plate washing and allowing wells semi-dry. A solution of enzyme substrate was added to all wells so as to establish the extent of binding between antiaflatoxin antibody and aflatoxin-enzyme conjugate whereby upon incubation color develops. The intensity of resulting color both in the sample and standard extracts was determined by reading absorbance at 450 nm using a spectrophotometer ELISA reader plate reader (Uniskan II_Finland). Aflatoxin levels were expressed in µg/kg, equivalent to parts per billion (ppb).

2.5 Data Analysis

Data analysis was based on General Lineal Model (GLM) suitable for unbalanced data using *PASW statistics* 18.0 for Windows software (SPSS Inc.) according to Payne et al.,[28]. Analysis of variance was performed on mean of variables including Moisture Content, fungal and aflatoxin load at 5% ($\alpha = 0.05$) significance level. Pearson's Chi-square test was used in the comparison of fungal and aflatoxin contamination frequencies according to farming system while Pearson's correlation established the relationship between ecological/ independent variable and biological/ dependent variables. Fungal and aflatoxin contamination constituted dependent variables while independent variables were moisture content and farming system.

3. RESULTS

3.1 Variation in Moisture Content (M.C) of Rain-Fed Farming System (RFFS) and Irrigation Farming System (IRFS) Maize Grain Samples Farming

The grains' moisture content for Rain Fed Farming System (RFFS) samples ranged from 11.1% to 14.8% with a mean of 13.2%, whereas the range for Irrigation Farming System (IRFS) samples was between 11.2% and 13.3% with a mean of 12.5%. An analysis of means showed that a significant difference existed between maize samples from the two farming systems (P < 0.001). The proportion of samples with M.C. \leq 13.5%, the recommended moisture content for safe storage of maize grain was comparatively higher for IRFS maize where all the 100% samples were safe for storage while for rain-fed samples it was 90% (Table 1).

3.2 Variation in Aflatoxigenic Fungal Load of Rain-Fed and Irrigated Maize Grain Samples

The maize samples in the two farming systems were found contaminated by the targeted aflatoxigenic fungal spp. including *A. flavus and A. parasiticus* at a relatively equal frequency of occurrence of 71.3% and 73.8% for RFFS and IRFS, respectively. However, *A. flavus* was more prevalent among RFFS than IRFS samples with 54.3% and 34.4%, of the samples contaminated, respectively. On the other hand *A. parasiticus* was more prevalent among IRFS compared to

 Table 1. Moisture content profiles for maize grain samples cultivated under rain-fed and irrigation farming system

Farming system	Highest M.C. (%)	Lowest M.C. (%)	Moisture content range (%)	Mean M.C. (%)	Proportion (%) of samples M.C ≤ 13.5%	
RFFS (n=94)	14.8	11.1	3.7	13.2±0.1	90	
IRFS (n=61)	13.3	11.2	2.1	12.5±0.1	100	

RFFS samples having occurred in 39.3% and 17.0% of the samples, respectively. However, the co-occurrence of both species was rare phenomena, whereby it was isolated in only 0.021% of RFFS samples but was lacking among IRFS samples (Table 2). In this study isolates having yellowish-green colony colors and smooth conidia were classified as *A. flavus* while those with dark-green colors and rough conidia were classified as *A. parasiticus*.

An analysis of the frequency of the two aflatoxigenic fungal species according to farming system established no significant differences $(X^2=16.764, P=0.05)$. The prevalence of *A. parasiticus* and *A. flavus* in IRFS samples were 46.7% and 53.3% of the contaminated samples, respectively. However, significant difference existed among RFFS samples where *A. flavus* was more prevalent than *A. parasiticus* occurring in 76.1% and 23.9% of the contaminated samples (Table 3).

The contamination levels by the aflatoxigenic fungi on account of total fungal load were in the range of 0-2000 CFU/g for rain-fed samples whereas for irrigation samples it was 0-600 CFU/g. The regional pattern regarding the relative distribution of the two aflatoxigenic spp. within the various levels of contamination established that *A. flavus* was more prevalent than *A. parasiticus* across all contamination levels in maize samples among rain-fed samples. In contrast, *A. parasiticus* was the predominantly isolated species among irrigation samples at similar contamination levels (Table 4).

A statistical analysis in the regional variation of means of total fungal load according to farming system established no significant difference (P < 0.001). The mean fungal load for RFFS samples was 83.5 CFU/g while IRFS samples had a mean of 130 CFU/g. However, the samples from both farming systems had contamination levels within the statutory limits of 1.0×10^5 CFU/g recommended by Kenya Bureau of standards.

3.3 Incidence of Aflatoxins in Maize Grain from Rain-Fed and Irrigation Farming System

The incidence of aflatoxins was analyzed in a total of 76 RFFS maize sample and all the 61 IRFS samples and the frequency of aflatoxin contamination was 73.7% and 59.0%, respectively. Among the contaminated samples, the highest proportion of samples had aflatoxin levels within the range > $4.0 \leq 10.0$ ppb. However, in both farming systems this proportion was approximately equal with 39.5% and 39.3% in RFFS and IRFS samples contaminated, respectively. Similarly, the proportion of samples that had more than 10 ppb of aflatoxins, the maximum statutorv contamination level recommended by Kenya Bureau of Standards was approximately the same for both farming systems with 17.1% and 16.4% among rain-fed and irrigation samples (Fig. 1).

The incidence of aflatoxins revealed that no significant differences existed according to farming system (P < 0.001.) The mean aflatoxin level was 12.8 ppb, 16.3 ppb and 15.7 ppb for Kitui and Kibwezi rain-fed samples and Perkerra irrigation samples, respectively.

3.4 Relationship between Maize Grains Moisture Content, Fungal Load and Aflatoxin Contamination

The relationship between the grains' moisture content and the fungal load and between total fungal load and aflatoxin levels in the maize samples in the two farming systems exhibited a positive correlation for both variables. However, a positively stronger correlation existed for rainfed than irrigation samples for both parameters

Table 2. Frequency of aflatoxigenic fungi in maize grain samples cultivated under rain-fed and irrigation farming system

Farming system	Samples (%)							
	Total aflatoxigenic fungi		A. flavus		A. arasiticus		Both spp.	
	AF ^a	RF ^b	AF ^a	RF⁵	RF^{b}	RF⁵	AF ^a	RF ^b
RFFS (n=94)	67	71.3	51	54.3	16	17.0	2	0.021
IRFS (n=61)	45	73.8	21	34.4	24	39.3	0)

^bRelative frequency (%)

with a correlation coefficient of R^2 =0.0192 and R^2 = 6.0 x10⁻⁶, respectively for the former relationship while for the latter relationship it was R^2 =0.0416 and R^2 = 0.0048, respectively. This weak relationship is clearly manifested by the statistical analysis by ANOVA (*P* < 0.001), whereby among the three independent variables under this study, only the mean moisture content had significant difference while the difference in the mean fungal load and aflatoxin contamination was insignificant according to the farming system (Fig. 2).

Table 3. Frequency of A. flavus andA. parasiticus in contaminated maize grainsamples cultivated under rain-fed andirrigation farming system

Farming system	Aflatoxigenic fungal species					
	A. flavus	A. parasiticus				
RFFS (n=67)	51 (76.1%)	16 (23.9%)				
IRFS (n=45)	24 (53.3%)	21 (46.7 %)				

4. DISCUSSION

4.1 Variation in Grain Moisture Content of Maize Cultivated under Rain-fed and Irrigation Farming Systems

Maize samples from rain-fed farming system had comparatively higher average moisture content than samples from irrigation farming system. Similarly, the highest moisture content recorded among all samples was also comparatively higher for rain-fed than irrigation samples. These observations were corroborated by the relatively higher proportion of irrigation samples with moisture content that was safe for maize grain storage. These differences in moisture content of maize grain from these two farming systems were supported by the statistical analysis which established that a significant difference existed.

The observed comparatively low moisture content in maize samples obtained from Perkerra irrigation scheme could be attributed to the strict planting and harvesting programme that farmers contracted by the Kenya Seed Company at Perkerra station are expected to adhere to. Maize cultivation in the scheme is undertaken under the supervision of Kenya Seed Company which provides farm inputs including certified seeds, fertilizer and also ready market for the harvest to be processed into commercial seeds. Drying of maize harvests to hinder fungal spoilage is undertaken over heavy gauge corrugated iron sheets after which the maize is ready for shelling and storage. These practices could therefore account for the low moisture content of maize samples cultivated under irrigation farming system unlike the situation in rain-fed farming region where such sound postharvest practices are not a routinely applied by farmers probably due to lack of economic incentives. The narrow moisture content range and fungal contamination and also the weak correlation between moisture content and fungal contamination among irrigation samples compared to rain-fed samples could also be on account of these sound farming practices.

4.2 Incidence of Aflatoxigenic Fungi and Aflatoxins in Maize Cultivated under Rain-fed and Irrigation Farming Systems

The relative similarity in frequency of both fungal and aflatoxin contamination according to farming system was statistically proven by analysis of these two variables where no significant difference was established. This observation is corroborated by other findings in this study whereby all the 155 maize samples from the two study regions had statutory safe fungal contamination levels recommended for human foods of 1.0 x 10⁵ CFU/g [17]. Further, the fact that over 80% of the samples from both farming systems had aflatoxins within the statutory limits according to Kenya Bureau of Standards implies that awareness of the dangers posed by fungal contamination of foods especially maize, is on the increase among Kenyans. This major finding is of great National significance regarding enhanced food safety, food security and public health.

The results on the relationship between grains' moisture content and fungal and aflatoxin contamination implies that though the difference in the mean moisture content of maize samples from these two farming systems was significantly different, it did not have great influence as to cause any significant difference in either the incidence of aflatoxigenic fungi or aflatoxins. This inference is further supported by the observation that despite a positive correlation in both farming systems between the moisture content and fungal load on one hand, and between fungal load and aflatoxin levels on the other hand, a weaker correlation was observed in the former compared to the latter combination of variables.

The significant relationship established between prevalence the various aflatoxigenic species and the farming system whereby *A. flavus* was

significantly more prevalent than A. parasiticus among the contaminated rain-fed samples are in agreement with previous research findings that established predominance of A. flavus over A.parasiticus in home-grown Kenyan maize [3]. Similarly, maize from other important maize producing regions of both East and West Africa have established A. flavus prevalence [29-31]. These findings also implies that factors including the prevailing agro-ecological and conditions, types of maize variety and irrigation practices at Perkerra scheme favor proliferation of both A. flavus and A. parasiticus while rain-fed farming, ambient climatic conditions and maize varieties grown is favorable for A. flavus in Kitui/ Kibwezi counties. Fundamentally, irrigation reduces the soil temperatures and the waterstress thereby hindering proliferation of aflatoxigenic fungi and subsequent aflatoxin at pre-harvest stage. The fungus *A. flavus* may also not compete well with other spp. under moist soils [32,33].

The main variable in this study was comparison of aflatoxigenic fungi and aflatoxins in maize grown under irrigation and rain-fed systems of cultivation. However, it's noteworthy to observe that while irrigation samples were obtained from fresh harvests, rain-fed samples were under storage conditions. The prevalence of *A. flavus* in both the two conditions in these findings therefore demonstrates that although it is typically considered a storage fungus, it is also a field fungus. The presence *A. flavus* in freshly harvested corn has previously been observed [34,35].

Table 4. Absolute and Relative frequencies (%) of aflatoxigenic fungi isolated from contaminated maize grain in rain-fed and irrigation samples according to level of fungal load (CFU/g)

Farming system	Mean CFU	Contamination range (CFU/g)	Total aflatox. fungi		A. flavus		A. parasiticus	
			AF ^a	RF⁵	AF ^a	RF⁵	AF ^a	RF⁵
RFFS	83.5±15	<u>></u> 500	6	9.0	5	7.5	1	1.5
(n=67)		<u>></u> 100<500	21	31.3	17	25.4	5	7.4
		<u>></u> 10<100	40	59.7	30	44.8	10	14.9
IRFS	130±16.3	>500	1	0.02	1	0.02	0	0
(n=45)		>100<500	31	68.9	14	31.1	17	37.8
. ,		>10<100	13	28.9	6	13.3	7	15.6

^aAbsolute frequency

^bRelative Frequency calculated from contaminated samples: Rain-fed=67:Irrigation=45 samples

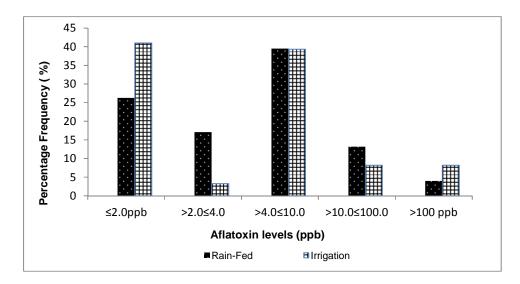


Fig. 1. Aflatoxin contamination profiles in maize grain samples from rain-fed and irrigation farming system according to various levels (ppb)

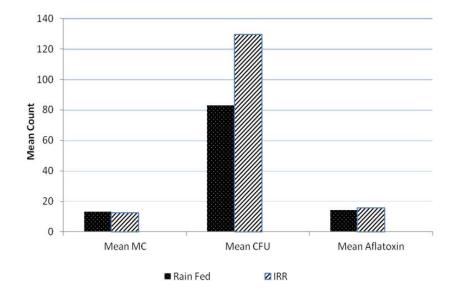


Fig. 2. Relationship between the mean moisture content, fungal load and aflatoxin levels in the maize cultivated under rain-fed and irrigation farming system

These new and interesting research findings implies that although irrigation farming system alleviates maize underproduction in Kenva, it does not necessarily significantly reduce harvests by contamination of the both aflatoxigenic fungi and aflatoxins as compared to rain-fed farming system. These observations are of special interest in allaying the general notion that only certain regions in Kenya are prone to epidemiological incidences of aflatoxin food poisoning. Instead, other regions with similar agro-ecological conditions and farming practices may have this problem as well. The counties historically associated with aflatoxicoses include Machakos, Kitui and the larger Makueni County from which Kibwezi sub-county was hived off from [3,4,12,36,37].

The major findings of this research therefore reveal that despite application of irrigation to enhance food security in Kenya, the maize harvests still had significant fungal and aflatoxin contamination. Further, in view of the fact that the maize varieties grown at Perkerra are high quality certified seeds supplied by Kenya Seed Company, the maize therefore would ideally have had significantly lower fungal and aflatoxin contamination levels than the maize samples from Kitui/Kibwezi counties where use of locally acquired seeds is a common practice [38-40]. Lack of this difference implies that factors other than primarily irrigation and post-harvest handling of the harvest affect fungal and aflatoxin incidence in these two farming systems.

Whereas irrigation may lower the soil temperatures, the air temperatures in maize fields still remains high. This condition, together with the humid atmosphere created by irrigation may provide ideal conditions for proliferation of aflatoxigenic fungi and subsequent aflatoxin contamination of maize cultivated Irrigation farming system at levels insignificantly different from Rain fed farming system. High aflatoxin levels have similarly been found in maize under irrigation in various regions including Benin, Mississippi delta and Southeastern United States [41-44]. However, in other related comparative experimental studies. significantly higher aflatoxin levels occurred in non-irrigated maize kernels relative to irrigated maize. The differences were even greater during years of lower than normal rainfall [45,46]. In other major food crops, similar findings have been established whereby peanuts cultivated under irrigation system in various parts of the world including Sudan and India have recorded lower aflatoxin contamination levels [47-49].

5. CONCLUSIONS

The findings from this study clearly reveal that despite from application of irrigation to enhance national maize production, post-harvest practices that prevent fungal and aflatoxin contamination should be incorporated into the entire maize production chain. This includes subsidizing cost of commercial maize seeds and installation of affordable grain driers in all the major maize producing regions and irrigation schemes. The most outstanding finding is that apart than regions historically associated with aflatoxin food poisoning in Kenya, the problem is a public health food hazard in other regions with similar agro-ecological conditions as well.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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